A Single Slide Multiplex Assay for the Evaluation of Classical Hodgkin Lymphoma

Denise Hollman-Hewley, MS, MBA,* Michael Lazare, BS,* Alex Bordwell, BS,* Emily Zebadua, MSc,* Pinky Tripathi, MBA,* Alexander S. Ross, PhD,* Deanna Fisher, BS,* Alisha Adams, BA,* Derek Bouman, BS,* Dennis P. O’Malley, MD,† and Lawrence M. Weiss, MD*†

Abstract: Classical Hodgkin lymphoma can be diagnosed with confidence in the majority of cases, but there is a significant subset that remains a diagnostic challenge. The authors have investigated the utility of a novel hyperplexing technology, MultiOmyx™, which may be applied to stain with >60 antibodies on single tissue sections from formalin-fixed paraffin-embedded tissue as an aid to the diagnosis of classical Hodgkin lymphoma. The multiplexing protocol included CD30, CD15, PAX-5, CD20, CD79a, CD45, BOB.1, OCT-2, and CD3 antibodies. The technology showed a high degree of sensitivity, specificity, and precision. Comparison studies with routine hematoxylin and eosin and immunohistochemical assessment of hematopathology cases in which classical Hodgkin lymphoma was included in the differential diagnosis showed concordance in 54 of 56 cases, with the 2 discordant cases illustrating the potential of this multiplexed immunofluorescence technology to improve on traditional immunohistochemistry for classical Hodgkin lymphoma diagnosis. This technology is practical for routine diagnosis and may be particularly useful in cases in which the sample size is limited, few Hodgkin-like cells are present, or in CD30-positive lymphoma cases with difficult morphology. MultiOmyx may potentially benefit other areas of research and diagnostic pathology.

Key Words: classical Hodgkin lymphoma, lymphoma diagnosis, hyperplex and multiplex staining, immunofluorescence, CD30

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Traditional or classical Hodgkin lymphoma is one of the success stories of modern oncology, with effective therapies approaching 90% five-year survival in low-stage patients successfully diagnosed and treated.1 Historically, Hodgkin lymphoma was diagnosed by the morphologic identification of Reed-Sternberg cells and variants (collectively Hodgkin cells) in the appropriate cellular milieu. Today, the availability of highly effective immunohistochemical (IHC) methodologies and antibodies reactive against formalin-resistant epitopes has refined the diagnosis of Hodgkin lymphoma and allowed for more accurate diagnosis, enabling its distinction from easily confused morphologic mimics such as T-cell histiocyte-rich large B-cell lymphoma, peripheral T-cell lymphomas with CD30-positive cells, and reactive cases with CD30-positive immunoblasts.2–4

Although the availability of effective IHC methodologies and antibodies has refined diagnosis, classical Hodgkin lymphoma remains one of the challenges in diagnostic pathology. First, its variable morphologic appearance can mimic a variety of lymphomas, other neoplasms, and reactive lymphadenopathies. Perhaps more importantly, although the overall immunophenotype of Hodgkin cells is characteristic, there is no single antibody that is diagnostic of classical Hodgkin lymphoma, and the pathologist must depend on analysis of a panel of antibodies of varying sensitivities and specificities to help establish the diagnosis.3–6 Complicating factors include the rarity of Hodgkin cells—typically <1%—and the frequency of other cells such as immunoblasts and histiocytes, which may be morphologic mimics and share one or more phenotypic findings with Hodgkin cells.17 For example, CD20 immunostaining of immunoblasts and CD15 immunostaining of histiocytes may be difficult to distinguish from staining on Hodgkin cells. In addition, clinical and economic pressures often mandate smaller biopsy specimens (usually core needle biopsy specimens) particularly common in hard-to-biopsy sites such as the mediastinum and the retroperitoneum, in which Hodgkin cells may be poorly represented or may “disappear” completely on deeper sections.

MultiOmyx, a novel hyperplexed fluorescence microscopy–based method, has been developed for the characterization of multiple analytes on single sections obtained from formalin-fixed paraffin-embedded tissue.18 In this technology, chemical inactivation of fluorescent
dyes after image acquisition allows reuse of common dyes in iterative staining and imaging cycles, and software can be used to compare the different stains in a practical setting familiar to a diagnostic pathologist. Whereas standard optical filters and multispectral imaging may resolve up to 5 to 7 separate fluorophores and therefore can be considered as a multiplex approach, this technology has been used to analyze the staining pattern of >60 protein antigens in single tissue sections, justifying the adoption of the term hyperplex to describe the capabilities of the system.

In the current study, we have examined the application of the novel technology, MultiOmyx, to the routine diagnosis of classical Hodgkin lymphoma using a multiplexing protocol including CD30, CD15, PAX-5, CD20, CD79a, CD45, BOB.1, OCT-2, and CD3 antibodies. We show that this technology is at least equivalent to routine morphologic and IHC evaluation of cases in which classical Hodgkin lymphoma is included within the differential diagnosis and holds great promise to be of added value in the diagnosis of cases with difficult morphology, few Hodgkin-like cells, or limited biopsy tissue.

MATERIALS AND METHODS

Tissue Specimens

Formalin-fixed paraffin-embedded lymph node excisions or large core needle biopsies were used. A total of 58 cases were obtained from referral cases received at Clarient Diagnostic Services Inc. (Aliso Viejo, CA) and deidentified, conforming to institutional standards for research. The historical diagnoses were obtained from the Clarient pathology reports, and the cases selected for study included cases of classical Hodgkin lymphoma, as well as cases considered in the differential diagnosis of classical Hodgkin lymphoma, including nodular lymphocyte-predominant Hodgkin lymphoma, B-cell lymphoma, T-cell/histiocyte-rich large B-cell lymphoma with CD30-positive cells, peripheral T-cell lymphoma with CD30-positive cells, and reactive lymph nodes with CD30-positive cells.

Historical IHC data from CD30, CD15, PAX-5, CD20, CD79a, CD45, BOB.1, OCT-2, and CD3 were available for comparison and benchmarking purposes. Furthermore, additional immunostains and Epstein-Barr encoding region (EBER) in situ hybridization data were available for review in a subset of the cases.

Reagents

The primary antibodies were obtained from the following sources: CD30 (NCL-L-CD30 clone 1G12; Leica Biosystems, Wetzlar, Germany), CD15 (OA952 clone C3D-1; Dako, Glostrup, Denmark), CD20 (1632 clone MS4A1; Epitomics, Burlingame, CA), CD45 (M0701 clone 2B11+PD7/26; Dako), CD3 (M7254 clone F7.2.38; Dako), CD79a (M7050 clone JCB117; Dako), polyclonal OCT-2 (sc-233, Santa Cruz Biotechnology, Dallas, TX), PAX-5 (AC-0158 clone EP156; Epitomics), and polyclonal BOB.1 (sc-955, Santa Cruz Biotechnology). Fluorescently tagged secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA): Cy3-Goat-anti-Mouse immunoglobulin (Ig)G (115-166-062) and Cy3-Goat-anti-Mouse IgM (115-165-075). For fluorescent conjugation of the primary antibodies, Cy3 and Cy5
N-hydroxysuccinimide (NHS) esters were used from GE Healthcare (PA23000, PA25000; Waukesha, WI).

**Antibody Labeling**

Seven primary antibodies (CD20, CD45, CD79a, CD3, BOB.1, OCT-2, and PAX-5) were conjugated with either Cy3 or Cy5 dyes for direct detection. If the antibody was not supplied in purified form, Protein A or Protein G HiTrap columns (GE Healthcare) were used following the manufacturer's protocols to purify the antibody before conjugation. After purification, the concentration was adjusted to 0.5 to 1.0 mg/mL, and the pH was adjusted to 8.2 to 9.0 with 1.0 M sodium bicarbonate to a final concentration of 0.1 M. A small amount of the NHS ester dye was dissolved in anhydrous dimethyl sulfoxide, and the concentration was determined by measuring the absorbance of a 1:250 dilution of dye stock in 1/2 phosphate-buffered saline (PBS) at the appropriate wavelength on an ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). A defined amount of reconstituted NHS dye was added to each reaction to yield a ratio of 2, 4, or 6 dye molecules per antibody, and the reaction was left in the dark for 60 minutes at room temperature. Upon completion of the reaction, the antibody solutions were buffer exchanged, and unbound dye was removed by Zeba desalting columns (Pierce, Rockford, IL). The antibody concentration and conjugation efficiency (dyes/antibody) were measured on the ND-2000 spectrophotometer.

**Multiplexing**

The multiplexing process consists of 3 primary steps: antibody staining, fluorescent image acquisition, and dye inactivation of the tissue. As there were a total of 9 antibodies in the assay, these multiplexing steps were repeated for a total of 5 rounds. The details of these steps have been described below.

**Initial Slide Preparation**

Before performing any of the multiplexing steps the slides were to undergo deparaffinization and rehydration; therefore, the formalin-fixed paraffin-embedded sections were placed in an oven at 60°C for 1 hour to initiate this process. Following this initial baking, the slides were transferred to a series of xylene and decreasing ethanol concentration washes. Once deparaffinized, antigen retrieval was performed at 110°C (20 min each) by means of a 2-step heat-induced epitope retrieval process that consisted of both acidic (citrate pH 6.0) and basic (Tris pH 9.0) solutions. Antibody clones that performed well using the 2-step antigen retrieval process were selected during antibody selection and validation process. The slides were then transferred to a 1× PBS wash and taken through several dye inactivation washes to minimize background autofluorescence. Upon completion, the slides were transferred to a solution of 3% bovine serum albumin (wt/vol) and 10% donkey serum (wt/vol) in 1× PBS to block any nonspecific binding. Finally, the slides were incubated in a diamidino-2-phenylindole solution for 15 minutes at room temperature and coverslipped.
with a 90% glycerol mounting medium that contained antifade reagents.

**Background Fluorescent Imaging**

The coverslipped samples were placed on a modified IN Cell Analyzer 2000 System (GE Healthcare), and a 10 × image was acquired in the diaminido-2-phenylindole, Cy3, Cy5, and fluorescein isothiocyanate channels. This background image served as a baseline for the native fluorescence of the tissue and was used in downstream image processing and subtraction. Upon completion of the imaging, the slides were decoverslipped so that the antibody stains could be applied. Decoverslipping was performed using a heated 1 × PBS water bath and gentle agitation.

**Antibody Staining**

All antibody staining reactions were performed for 1 hour at room temperature on the Leica Bond III automated stainer. The antibody staining sequence was as follows: round 1, CD30/BOB.1; round 2, CD15/OCT-2; round 3, CD20/PAX-5; round 4, CD45/CD3; round 5, CD79a (Fig. 1). Each antibody pair was empirically determined to ensure there was no antibody-antibody cross-reactivity by comparing the staining of cocktailed antibodies with their respective individual antibody staining. In the cases in which direct detection was used, the Cy3/Cy5 conjugates were cocktailed together and applied simultaneously to the tissue. For CD30 and CD15 applications, indirect detection was used, and hence the secondarily fluorescent conjugated antibodies were cocktailed with the direct conjugates for those rounds. For CD30, direct conjugate staining resulted in lower intensity compared with the unconjugated CD30 antibody. As direct conjugation can potentially perturb antigen binding affinity for certain antibodies, this was not an unexpected finding. As CD30 is critical for staining Reed-Sternberg cells, indirect detection was used to ensure maximum staining intensity. For CD15, the conjugation method used in this study was not compatible with its IgM isotype, and therefore indirect detection was utilized. Upon completion of the antibody staining, the slides were decoverslipped with a 90% glycerol mounting medium that contained antifades and then transferred to the IN Cell Analyzer 2000 System for imaging.

**Fluorescent Imaging**

Fluorescent images of the tissue were acquired after each antibody staining round and after the tissue underwent dye inactivation. A × 10 objective was used for both the background imaging step and after the first round of antibody staining with CD30. The CD30 image was then presented to the pathologist, who used this to select regions of interest (ROIs) (Fig. 2). These ROIs defined the locations for the higher magnification (× 40 imaging) not only for CD30 but also for the remaining 8 biomarkers. Upon completion of the imaging, the slides were decoverslipped, allowing for the next round of antibody application or dye inactivation of the tissue.

**Dye Inactivation of Tissue**

Decoverslipped slides were washed in a 1 × PBS solution and then transferred to an alkaline solution containing H₂O₂ for 15 minutes at room temperature, as described by Can et al. Upon completion, the slides were coverslipped and placed on the IN Cell Analyzer 2000 System for imaging as highlighted above.

**Image Processing**

After each imaging round, 2 processing methods were applied to the images. One was for the round-to-round registration of the images to ensure alignment of the tissue and to allow for direct interpretation of the antibody staining on a cell-by-cell basis. The second processing method was to remove the inherent autofluorescence of the formalin-fixed paraffin-embedded tissue, allowing for the antibody fluorescent signal to be separated from the background fluorescence. Both of these methodologies are described in detail in Gerdes et al.

**Interpretation and Comparison of the Multiplexed Data With Traditional IHC**

Images from all 9 biomarkers were presented to the pathologist as either standard monochromatic grayscale images or algorithmically generated molecular 3,3'-diaminobenzidine (DAB) images. Molecular DAB images appear similar to traditional brown, brightfield IHC images but are generated from the fluorescence data; this algorithmic transformation is described by Kenny. Using the molecular DAB images, the pathologist rendered a diagnosis for each case as either representing classical Hodgkin lymphoma or not classical Hodgkin lymphoma (ie, whether the multiplex immunofluorescence could rule in or rule out a diagnosis of classical Hodgkin lymphoma). These diagnoses were then benchmarked to the historical diagnoses obtained from the traditional methods.

**Statistics Tests**

A Fisher exact test was used to compare the diagnosis achieved with the multiplexed immunofluorescence assay versus the historical diagnosis. One-way analysis of variance (ANOVA) was used to assess repeatability of the semiquantitative scoring of each antibody. All P values reported are 2-sided, with a P value of < 0.05 considered significant.
significant. A standard repeatability score ($r$), using an ANOVA framework, assessed repeatability as the fraction of variance between patients compared with the total variance (including repeated measurements). The score was measured as $S_A^2/(S_A^2 + S_B^2)$, in which $S_A^2$ is the between-groups variance and $S_B^2$ is the within-group variance.

**RESULTS**

Fifty-eight cases were originally selected for study. Two cases were eliminated on the basis of having only 1 CD15 field available for review. The remaining 56 cases included 25 cases of classical Hodgkin lymphoma; 5 cases of nodular lymphocyte-predominant Hodgkin lymphoma; 11 cases of B-cell lymphoma, including 3 cases of T-cell/histiocyte-rich large B-cell lymphoma; 7 cases of peripheral T-cell lymphoma, including 3 cases of anaplastic large cell lymphoma; and 8 cases of reactive lymph nodes with CD30-positive cells.

In general, the computer-generated images obtained with the immunofluorescence studies were comparable to routine IHC studies. In addition, this novel hyperplexing technology allowed for direct comparison of stains on the

same tissue section (Fig. 3, showing 1 Hodgkin cell with the left stained for CD30 and the right stained for CD45). Thus, it was possible to determine comprehensive immunophenotypes on individual cells, enabling specific assessment of the CD30-positive population and distinguishing staining of other antibodies on other cell types, such as CD15 staining of histiocytes.

A subset of 24 specimens underwent comprehensive antibody specificity studies. The individual antibody specificity was found to be 100% in all but 1 of the 9 markers assessed for each case. In 1 case, CD30 staining was found to be nonspecific by immunofluorescence. Qualitative comparison between immunofluorescence and IHC studies showed comparability for most antibodies, although CD15 perhaps showed stronger staining, and BOB.1 and OCT-2 showed weaker staining by immunofluorescence in a subset of cases. Six specimens underwent intraday precision studies, performed by the same individuals on the same instruments with the same reagents in triplicate; 100% concordance of staining was seen. Five

specimens underwent interday precision studies, performed by different individuals on different instruments with different reagents in triplicate; again, 100% concordance of antibody staining was seen.

The results of the multiplex immunofluorescence studies and the historical pathologic diagnosis were concordant in 54 of the 56 cases ($P < 0.0001$ for the Fisher exact test) (Figs. 4, 5). One case had been historically diagnosed as classical Hodgkin lymphoma, yet it was classified by the multiplex immunofluorescence studies as B-cell lymphoma on 6 separate occasions (as this particular case was part of the precision studies). Review of the original hematoxylin and eosin and IHC studies raised doubt as to the validity of the historical diagnosis (L.M.W., personal observations) (Fig. 6). The second case had been diagnosed as T-cell lymphoma, anaplastic large cell type, versus classical Hodgkin lymphoma (Fig. 7). This case was included in the study because a subsequent biopsy performed 2 weeks later was diagnosed as anaplastic large cell lymphoma. Therefore, the results from the MultiOmyx study on the initial specimen were concordant with the diagnosis.

The cases were also scored on a semiquantitative scale of 0 to 3 for each antibody. Considering only stains deemed evaluable, a 1-way ANOVA was used to assess repeatability of the markers on this scale (Table 1). When all repeated studies were examined, an $F$ statistic was significant for all markers, indicating low variability between repeats. Within the 6 specimens that underwent intraday precision studies using the same antibody lot, 2 markers did not show significance because of the low variability of these markers between the 6 selected cases (primarily nonclassical Hodgkin lymphomas, data not shown). The repeatability score, $r$, varied from 0.44 to 0.91 for the markers when all repeated studies were assessed.

![Case originally diagnosed as classical Hodgkin lymphoma by routine morphology and IHC reclassified as B-cell lymphoma (T-cell/histiocyte-rich large B-cell lymphoma) on the basis of the hyperplexed immunofluorescence technology. The morphology is shown in (A), highlighting Hodgkin-like cells. Although positive for CD30 (B) and negative to focally positive for CD45 (C), this case was negative for CD15 (D) by both IHC and immunofluorescence and was also positive for multiple B-lineage markers, including PAX-5 (E), CD20 (F), CD79a (G), BOB.1 (H), and OCT-2 (I).](image-url)
DISCUSSION

Mandatory review of pathology material before the treatment of patients with lymphoma can identify a significant number of misclassified cases for which the revised diagnosis results in a therapeutic change. A recent study from the University of Nebraska Medical Center revealed that in 15% of lymphoma patients, secondary review led to revised diagnosis, resulting in a change of therapy in most of the cases. In our study, we report the application of a novel hyperplexing immunofluorescent technology (ie, suitable for staining >60 antibodies on a single tissue section), MultiOmyx, for the routine diagnosis of classical Hodgkin lymphoma, with the potential to aid pathologists in the differential diagnosis of CD30-positive lymphoma cases. We chose a panel of 9 antibodies to distinguish Hodgkin cells from other cell types and, therefore, classical Hodgkin lymphoma from other diagnoses. Maintaining the panel at 9 markers minimized the theoretical risk for steric hindrance at later rounds of staining. Throughout the MultiOmyx process, antibodies remained bound after each round of staining and dye inactivation. To address concerns related to potential steric hindrance at later rounds, and to determine the final multiplexing order, experiments were carried out to show equivalence specificity and sensitivity between single-plexed and multiplexed staining across multiple rounds. Tissue loss was not observed with multiple rounds of immunostaining and dye inactivation on the 9 Hodgkin markers, and MultiOmyx processing has been successfully demonstrated in up to 61 markers. CD30 was chosen because of its high sensitivity for Hodgkin cells and was used to select fields of interest in individual cases for further study. CD15 and PAX-5 were also chosen for their sensitivity for Hodgkin cells, CD20, CD79a, CD45, BOB.1, and OCT-2 were chosen because of their selective reactivity on nodular lymphocyte-predominant Hodgkin lymphoma, B-cell lymphoma, and reactive immunoblasts and their lower frequency of expression in classical Hodgkin lymphoma, whereas CD45 and CD3 are markers of peripheral T-cell lymphoma (albeit imperfect ones) and are rarely expressed on Hodgkin cells. Although a case could be made for the addition of other antibodies (eg, CD43, EBV-latent membrane protein, MUM-1, etc.), we attempted to keep the panel to a practical size for routine diagnosis with acceptable turnaround time. It is possible, however, to envision the validation of a full panel of hematopathology markers, including in situ hybridization probes such as EBER.

The visualization of the immunofluorescent staining can either be monochromic, polychromic, or mimicking routine IHC (“molecular” DAB). The latter was still compatible with direct comparison of different stains, as they could be visualized either by viewing serial images on the computer screen, multiple views of the same field on the same screen, or by splitting the screen to view different images on different parts of the screen using our proprietary viewer. Using this technology, it is possible to distinguish staining of antibodies on CD30-positive cells from staining of morphologically similar cells. For example, CD15 staining of CD30-positive Hodgkin cells could be distinguished from CD30-negative histiocytes.
and CD79a/BOB.1/OCT-2-positive immunoblasts could be distinguished from CD79a/BOB.1/OCT-2-negative (typically) Hodgkin cells.

This technology was found to be at least equivalent to traditional hematoxylin and eosin and IHC, but numerous circumstances can be envisioned in which the hyperplexing immunofluorescent technology might have inherent advantages. This would include small needle core biopsy specimens, as are often obtained in the evaluation of mediastinal or retroperitoneal biopsies, cases with few Hodgkin-like cells for evaluation, cases in which it is difficult to assess antigen expression on Hodgkin-like cells by traditional IHC, and limited number of available slides for immunostaining (consultation material, or specimen almost cut through). Currently specialized technical expertise is needed, but widespread use is envisioned leading to the successful adoption for routine diagnostic work. There are some potential barriers as with any new technology. First, in contrast to routine microscopy, the images are reviewed on computer monitors. There are challenges inherent to this type of evaluation, as evidenced by literature from telepathology. These barriers, although real, can be surmounted as more experience and training in the use of “virtual” microscopy is gained in all areas of pathology. Second, the pathologist must become comfortable selecting and analyzing ROIs (necessary to decrease processing time), as opposed to whole tissue sections. This may not be a factor in the evaluation of small needle biopsies, in which it may still be practical to analyze the entire sample. Finally, the pathologist must learn to integrate the findings from this novel technology with clinical, morphologic, and routine IHC findings; this technology alone will not generate a specific diagnosis but will be an aid, as other technologies, to the diagnostic pathologist. This is similar to the previous incorporation of flow cytometry findings into routine histologic diagnosis.

In conclusion, the novel hyperplexing technology, MultiOmyx, holds promise as a practical aid in the routine diagnosis of Hodgkin lymphoma and, by analogy, may be applied to other areas of research and diagnostic pathology. This technology will be of particular advantage in assessing limited-size samples with limited cells of interest, as well as situations in which there is significant cell-to-cell heterogeneity. Study of cell-to-cell heterogeneity in cancer and other diseases has only recently been addressed in scientific analyses, and multiplexed immunofluorescence studies have definite potential for furthering our understanding of disease pathogenesis and for precision medicine approaches.

REFERENCES


